CRYSTALS OF THE SODIUM SALT OF PRAVASTATIN

Patent Number: WO0110813
Publication date: 2001-02-15
Inventor(s): PFLAUM ZLATKO (SI)
Applicant(s):: LEK TOVARNA FARMACEVTSKIH (SI); PFLAUM ZLATKO (SI)
Requested Patent: SI20305
Application Number: WO2000IB01103 20000804
Priority Number(s): SI19990000191 19990806
IPC Classification: C07C69/732 ; C07C67/52 ; A61K31/22
EC Classification: C07C69/732
Equivalents:

Abstract

Lovastatin, pravastatin, simvastatin, mevastatin, atorvastatin, fluvastatin and cervastatin and derivatives and analogs thereof are known as HMG-CoA reductase inhibitors and are used as antihypercholesterolemic agents. The majority of them are produced by fermentation using microorganisms of different species identified as species belonging to *Aspergillus, Monascus, Nocardia, Amycolatopsis, Mucor* or *Penicillium* genus, some are obtained by treating the fermentation products using the methods of chemical synthesis or they are the products. The present invention relates to a novel crystalline form of the sodium salt of pravastatin, which is known by the chemical name 1-naphthaleneheptanoid acid, 1, 2, 6, 7, 8, 8a-hexahydro- beta , delta , 6-trihydroxy-2-methyl-8 (2-methyl-1-oxobutoxy)-, mono sodium salt, which is useful as a pharmaceutical substance, to the method for its production and isolation, to a pharmaceutical formulation containing the crystalline form of the sodium salt of pravastatin and a pharmaceutical method of treatment. The novel crystalline form of the sodium salt of pravastatin is useful in the treatment of hypercholesterolemia and hyperlipidemia.

Description

CRYSTALS OF THE SODIUM SALT OF PRAVASTATIN

The present invention relates to a crystalline form of the sodium salt of pravastatin, which is known by the chemical name 1-naphthaleneheptanoid acid, 1,2,6,7,8,8ahexahydro-P,b,6-trihydroxy-2-methyl-8- (2-methyl-l- oxobutoxy)-, mono sodium salt, which is useful as a pharmaceutical substance. The present invention further relates to the method for its preparation and isolation, to a pharmaceutical formulation containing the sodium salt of pravastatin in the crystalline form and a pharmaceutically acceptable carrier, and to the pharmaceutical method of treatment.

Lovastatin, pravastatin, simvastatin, mevastatin, atorvastatin, fluvastatin and cervastatin and derivatives and analogs thereof are known as HMG-CoA reductase inhibitors and are used as antihypercholesterolemic agents. The majority of them are produced by fermentation using microorganisms of different species identified as species belonging to Aspergillus, Monascus, Nocardia, Amycolatopsis, Mucor or Penicillium genus, some are obtained by treating the fermentation products using the methods of chemical synthesis (simvastatin) or they are the products of total chemical synthesis (fluvastatin, atorvastatin and cervastatin)

Processes for the preparation of the sodium salt of pravastatin in a solid form known from the prior art comprise, for example, the step of lyophilisation. After lyophilisation only the solvent is removed but impurities remain together with the sodium salt of pravastatin.

Apart from the aforementioned, lyophilisation is not very economical in large-scale production operations. During precipitation due to nonselectivity of the process, impurities precipitate together with the desired substance. Compared to the both aforementioned processes for the preparation of pharmaceutical substances in the solid form, crystallization is the only selective process wherein the molecules of the desired substance are selectively incorporated into the crystal matrix.

Possibility of inclusion of impurities into the crystal is minimal because only small size molecules are able to incorporate into intermolecular space inside a crystal (related impurities, which are usually within the desired substance size range may only be incorporated into this space with great difficulty). incorporation of other molecules into the crystal matrix is not favoured thermodynamically.

The advantage of substances in the crystal structures over those in amorphous structures is that their physical as well as chemical parameters are better defined and they are more stable. The latter is of particular importance for the substances which in their nature are unstable and sensitive to different ambient influences, such as light, pH, atmosphere and temperature.

Pravastatin sodium is particularly sensitive to these negative influences. (light, pH, atmosphere and temperature)

It has been known that thus far the sodium salt of pravastatin may only be present in an amorphous form. The Merck Index 1996 describes the sodium salt of pravastatin as an amorphous substance.

Methods for the preparation of the sodium salt of pravastatin described in many patents, for example US Pat. No. 44,537,859, US Pat. No. 4,448,979, US Pat. No.

4,410,629 and US Pat. No. 4,346,227, afford only the preparation of an amorphous form. In the methods disclosed, after separation on the

is obtained.

The WO-A-98/45410 discloses that after the sodium salt of pravastatin is purified using reverse-phase chromatography, alleged crystals may be obtained by precipitation in the ethanol/ethyl acetate mixture; however, the experiments we have carried out suggest that this combination of the solvents affords the preparation of pravastatin in the amorphous form and not in the crystalline form.

It is an object of the present invention to provide the sodium salt of pravastatin which is improved in purity and stability compared to the salts described in the prior art mentioned above.

Further, it is another object of the present invention to provide a process for the preparation of such a sodium salt of pravastatin.

These and further objects are accomplished by the present invention.

In a first aspect, the present invention provides the sodium salt of pravastatin in a crystalline form.

Furthermore, the present invention also provides the sodium salt of pravastatin in a specific crystalline form, wherein the crystals in an X-ray diffraction measurement produce a signal sufficiently comparable to that illustrated in the diffractogram shown in Figure 2.

In a second aspect, the present invention provides a process for the preparation of the sodium salt of pravastatin in a crystalline form comprising the steps of: (a) dissolution of the sodium salt of pravastatin in a lower aliphatic alcohol ; (b) addition of ethyl acetate to the alcoholic solution of the sodium salt of pravastatin; (c) cooling of said alcohol/ethyl acetate mixture; and (d) crystallization.

According to a third aspect of the present invention, there is further provided a pharmaceutical formulation containing the sodium salt of pravastatin in the aforementioned crystalline forms.

The crystalline sodium salt of pravastatin according to the present invention is particularly suitable for the preparation of pharmaceutical products for the treatment of hypercholesterolemia and hyperlipidemia.

In the following, drawings will be briefly described.

Figure 1: Diffractogram of a conventional amorphous sodium salt of pravastatin which is commercially available, scanned on the X-ray powder diffractometer within 2 to 42 2θ range with a 0 025 2θ step and an integration time of 1 second/step.

Figure 2: Diffractogram of crystals of the sodium salt of pravastatin prepared according to Example 2 of the present invention, which are scanned on the X-ray powder diffractometer within 2 to 48 2θ range with a 0 035 2θ step and an integration time of 1 second/step.

Figure 3: Image of the amorphous sodium salt of pravastatin used for the X-ray diffraction measurement shown in Fig. 1, which is obtained under the microscope under 400-fold magnification.

Figure 4: Image of crystals of the sodium salt of pravastatin prepared according to Example 2 of the present invention, obtained under the microscope under 400-fold magnification.

X-ray diffraction measurements were carried out with a X-ray powder diffractometer (Phillips PW 1710) using a Cu-Ka (20 mA, 40 kV, X = 1.5406 A) light source. For microscopic observations, an OLYMPUS BX 50F microscope with a CCD Sonny DXC-950-P camera was used with 400-fold magnification.

In the following, the present invention will be illustrated in more detail by the description of preferred embodiments.

In our research work we have surprisingly found that by suitable selection of the solvents and adequate order of their use the sodium salt of pravastatin in a form having an improved crystallinity, relative to the conventional solid form, can be prepared. Thus, in contrast to the while appearance of the pravastatin sodium solid described in the WO-A-98/45410, it is possible according to the present invention to achieve crystals exhibiting a colorless or pale yellow appearance, which clearly indicates the improved crystallinity and, thus, the clearly crystalline form of the sodium salt of pravastatin provided by the present invention.

Other physical properties of the crystals of the pravastatin sodium of the present invention further indicate the improved crystallinity obtained. Firstly, the crystals according to the present invention can be preferably obtained in the form of needles, sometimes in the form of radiating clusters. Such crystal shape can be readily observed under the microscope, for example when the observation is carried out under 400-fold magnification (see Fig. 4). By contrast, conventionally available amorphous pravastatin sodium appear in the shape of granular particles (see Fig. 3).

Secondly, the melting point of the pravastatin sodium crystals according to the present invention is preferably between 170 and 174 C, more preferably between 172 and 174 C. This melting point range achieved in the present invention is very small for such a complicated chemical structure and confirms the high crystallinity of the pravastatin sodium crystals obtained.

Thirdly, a further characteristic feature of the crystalline pravastatin sodium according to the present invention is that the signal obtained in an X-ray diffraction measurement (Cu-Ka, 2θ) have sharp and distinct peaks. In particular, the shape of the X-ray diffraction peaks of pravastatin sodium according to the present invention are defined by a small half-value width, which confirms a high degree of crystallinity. The term"half-value width"means the value of the 2θ range of one peak at the half height or magnitude of the respective peak. Accordingly, the signals obtained by these measurements comprise distinct peaks (2θ) having a half-value width preferably below 2 , more preferably below 1 , and most preferably below 0,5
Exemplary crystals of pravastatin sodium prepared according the present invention produce a diffractogram in an X-ray diffraction measurement that is shown in
Figure 2. Due to its particularly improved crystallinity and, thus, purity and stability, such pravastatin sodium crystals which, in an X ray diffraction measurement (Cu-Ka, 2θ), produce a signal sufficiently comparable to that, preferably essentially as that illustrated in the diffractogram shown in Figure 2

constitute preferred embodiments of the present invention. Unit cells of this crystal could not be determined because of its size and high background at the angles > 20 20. Comparison of the recorded diffractogram with the reference from the PDF and CSD databases (PDF-"Powder Diffraction File issued by"International Center for Diffraction Data", 12 Campus
Boulevard, Newtown Square, PA 19073-3273 USA; CSD "Cambridge Structural Database System"issued by "Cambridge Crystallographic Data Centre", 12 Union Road,
Cambridge CB2 IEZ, the United Kingdom) has shown that the crystals of the sodium salt of pravastatin according to the present invention are really a novel and thus only known crystalline form of the sodium salt of pravastatin.

For comparison, a diffractogram of amorphous pravastatin sodium which is commercially available is shown in Figure 1.

The process for the preparation of crystals according to the present invention as described above comprises the following steps: (a) Providing a solution containing pravastatin and sodium cations in a lower aliphatic alcohol. This is suitably carried out by dissolution of an solid and/or amorphous sodium salt of pravastatin in a lower aliphatic alcohol having preferably 1 to 4 carbon atoms. More preferably, the alcohol used for the dissolution of pravastatin sodium is ethanol or methanol. The best crystallization results have been achieved when preparing a solution of pravastatin sodium in methanol.

(b) Addition of ethyl acetate into the alcoholic solution, preferably while the alcoholic solution obtained in step (a) is stirred continually. The addition of ethyl acetate into the alcoholic solution of pravastatin sodium is preferably carried out slowly, while the addition may be continuously or stepwise

(c) Cooling of the resulting alcohol/ethyl acetate mixture

(d) Crystallization

In step (d), from the cooled mixture crystals of the sodium salt of pravastatin, which preferably have a colorless or pale yellow appearance and are in the form of needles or radiating clusters, are formed.

Additionally, the crystals obtained by this process may preferably be filtered, ethyl acetate washed and dried.

The crystallization is carried out advantageously if the initial concentration of the sodium salt of pravastatin in the aliphatic alcohol used for the dissolution is preferably between 0.03 and 0.3 g/ml, more preferably between 0.05 and 0.2 g/ml, particularly about 0.1 g/ml, and if the volume of added ethyl acetate in step (b) does preferably not exceed the 15-fold volume, more preferably the 10-fold volume of the starting solution of the sodium salt of pravastatin in the aliphatic alcohol.

Furthermore, to achieve a higher crystallization rate, the preferred temperature of crystallization is below 15 C, more preferably below 10 C, particularly about 8 C.

For enforcing further crystallization, it is preferred to carry out the process according to the invention with additional steps of (e) Further adding ethyl acetate to the mixture of step (d). This is done after an appropriate period of a first crystallization stage where crystallization occurs

(f) then, crystallization of pravastatin sodium is continued while cooling.

With such an additional crystallization stage the yield of crystalline pravastatine sodium can be increased, normally by 5 to 10 %.

The volume of ethyl acetate additionally added to the cooled mixture in step (e) is preferably in the range of from 25 to 75 % by volume, more preferably from 40 to 60 % by volume based on the volume of ethyl acetate added in step (b).

Furthermore, the crystals are preferably formed within a total crystallization time of 3 to 20 hours. More preferably, the total crystallization time is between 4 and 12 hours, particularly about 4 hours.

The present invention also relates to pharmaceutical formulations containing the sodium salt of pravastatin in the form of crystals. The pharmaceutical formulation is present in the form which is suitable for oral and parenteral administration, respectively, and is useful for the treatment of hypercholesterolemia and hyperlipidemia. The pharmaceutical formulation of the present invention is available in the form of tablets, capsules, granules and suppositories as well as in the form of suspensions.

The pharmaceutical formulation of this invention may comprise, in addition to the sodium salt of pravastatin, one or more fillers, such as microcrystalline cellulose, lactose, sugars, starches, modified starch, mannitol, sorbitol and other polyols, dextrin, dextran and maltodextrin, calcium carbonate, calcium phosphate and/or hydrogen phosphate, sulphate, one or more binders, such as lactose, starches, modified starch, dextrin, dextran and maltodextrin, microcrystalline cellulose, sugars, polyethylene glycols, hydroxypropyl cellulose, hydroxypropyl methylcellulose, ethylcellulose, hydroxyethyl cellulose, methylcellulose, carboxymethyl cellulose, gelatin, acacia gum, tragacanth, polyvinylpyrrolidone, magnesium aluminium silicate, one or more disintegrating agents such as croscarmellose sodium, cross-linked polyvinylpyrrolidone, cross-linked carboxymethyl starch, starches and microcrystalline cellulose, magnesium aluminium silicate, polyacrylin potassium, one or more different glidants such as magnesium stearate, calcium stearate, zinc stearate, calcium behenate, sodium stearyl fumarate, talc, magnesium trisilicate, stearic acid, palmitic acid, carnauba wax, silicon dioxide, one or more buffering agents such as sodium or potassium citrate, sodium phosphate, dibasic sodium phosphate, calcium carbonate, hydrogen phosphate, phosphate, sulphate, sodium or magnesium carbonate, sodium ascorbinate, benzoate, sodium or potassium hydrogen carbonate, lauryl sulphate, or mixtures of such buffering agents.

If required any, the formulation may also comprise surfactants and other conventional components for solid, pharmaceutical formulations such as coloring agents, lakes, aromas and adsorbents. As surfactants the following may be used: ionic surfactants, such as sodium lauryl sulphate or non-ionic surfactants such as different poloxamers (polyoxyethylene and polyoxypropylene copolymers), natural or synthesized lecithins, esters of sorbitan and fatty acids (such as Spano, manufactured by Atlas Chemie), esters of polyoxyethylenesorbitan and fatty acids (such as Tween@, manufactured by Atlas Chemie), polyoxyethylated hydrogenated castor oil (such as Cremophoro, manufactured by BASF), polyoxyethylene stearates (such as Brio, manufactured by Atlas Chemie), dimethylpolysiloxane or any combination of the above mentioned surfactants.

If the solid pharmaceutical formulation is in the form of coated tablets, the coating may be prepared from at least one film-former such as hydroxypropyl methylcellulose, hydroxypropyl cellulose, at least from one plasticizer such as polyethylene glycols, dibutyl sebacate, triethyl citrate, and other pharmaceutical auxiliary substances conventional for film coatings, such as pigments, fillers and others.

The pharmaceutical formulation can be prepared by conventional formulation methods known to those skilled in the art.

The present invention is illustrated but by no means limited by the following examples.

Example 1
The sodium salt of pravastatin (1 g) was dissolved in methanol (10 ml) and while stirring ethyl acetate was added. The resulting clear yellow solution was cooled to 8 C and allowed to stand overnight. Formed radiating clusters of thin, long needle-like crystals were filtered, washed with ethyl acetate (20 ml) and dried.

Yield: 0.87 g of pale yellow crystals, melting point 172-174 C.

Example 2
The sodium salt of pravastatin (2 g) was dissolved in methanol (20 ml) and while stirring ethyl acetate (80 ml) was added. The clear, slightly yellow solution was cooled to 8 C and allowed to stand for 4 hours. Formed radiating clusters of thin, long needle-like crystals were filtered, washed with ethyl acetate (20 ml) and dried.

Yield: 1.53 g of colorless crystals, melting point 172-174 C.

Example 3
The sodium salt of pravastatin (2 g) was dissolved in methanol (20 ml) and while stirring ethyl acetate (150 ml) was added. The resulting clear, slightly yellow solution was cooled to 8 C and allowed to stand for 4 hours. Formed radiating clusters of thin, long needlelike crystals were filtered, washed with ethyl acetate (20 ml) and dried. Yield: 1.66 g of colorless crystals, melting point 172-174 C.

Example 4
The sodium salt of pravastatin (2 g) was dissolved in methanol (20 ml) and while stirring ethyl acetate (170 ml) was added. The resulting clear, slightly yellow solution was cooled to 8 C and allowed to stand for 4 hours. Formed radialing clusters of thin, long needlelike crystals were filtered, washed with ethyl acetate (20 ml) and dried. Yield: 1.75 g of colorless crystals, melting point 172-174 C.

Example 5
The sodium salt of pravastatin (2 g) was dissolved in methanol (12 ml) and while stirring ethyl acetate (100 ml) was added. The resulting clear, slightly yellow solution was cooled to 8 C and allowed to stand for 1 hour. After that further ethyl acetate (60 ml) was added, so the pravastatin still dissolved in the solution was forced to crystallize. After 2 hours at 8 C the formed radiating clusters of thin, long needle-like crystals were filtered, washed with ethyl acetate (20 ml) and dried. Yield: 1.85 g of colorless crystals, melting point 172-174 C.

Claims

Claims 1. The sodium salt of pravastatin in a crystalline form.

2. The sodium salt of pravastatin according to claim 1, wherein the crystals exhibit a colorless or pale yellow appearance.

3. The sodium salt of pravastatin according to claim 1 or claim 2, wherein the crystals clearly appear in the form of needles or radiating clusters.

4. The sodium salt of pravastatin according to any one of claims 1 to 3, wherein the melting point is in the range of from 170 C to 174 C.

5. The sodium salt of pravastatin according to any one of claims 1 to 4, wherein the crystals in an X-ray diffraction measurement produce distinct peaks (29) having a half-value width below 2 .

6. The sodium salt of pravastatin in a crystalline form, wherein the crystals in an X-ray diffraction measurement produce a signal sufficiently comparable to that illustrated in the diffractogram shown in Figure 2.

7. A process for the preparation of the sodium salt of pravastatin in a crystalline form, comprising the steps of: (a) providing a solution containing pravastatin and
sodium cations in a lower aliphatic alcohol; (b) addition of ethyl acetate to said alcoholic solution; (c) cooling of said alcohol/ethyl acetate mixture ; and (d) crystallization.

8. A process according to claim 7 additionally comprising after a first crystallization stage the steps of: (e) addition of further ethyl acetate to the alcohol/ethyl acetate mixture; and (f) further crystallization.

9. A process according to claims 7 or 8, wherein the lower aliphatic alcohol is ethanol or methanol.

10. A process according to claims 7 or 8, wherein the lower aliphatic alcohol is methanol.

11. A process according to any one of claims 7 to 10, wherein the addition of ethyl acetate is exhibited while the alcoholic solution of the sodium salt of pravastatin is stirred continually.

12. A process according to any one of claims 7 or 11, wherein the concentration of the sodium salt of pravastatin in the alcoholic solution of step (a) is between 0.03 and 0.3 g/ml.

13. A process according to any one of claims 7 to 12, wherein the volume of added ethyl acetate in step (b) does not exceed the 15 fold volume of the initial alcoholic solution of the sodium salt of pravastatin.

14. A process according to any one of claims 8 to 13, wherein the volume of further added ethyl acetate in step (e) is in the range of from 25 to 75 % by volume based on the volume of ethyl acetate added in step (b).

15. A process according to any one of claims 7 to 14, wherein the alcohol/ethyl acetate mixture is cooled to a temperature below 15 C.

16. A process according to any one of claims 7 to 15, wherein the total crystallization time is between 3 and 20 hours.

17. A process according to any one of claims 7 to 16, wherein the formed crystals are filtered, ethyl acetate washed and dried.

18. A pharmaceutical formulation containing the sodium salt of pravastatin in a crystalline form.

19. The pharmaceutical formulation according to claim 18 containing the sodium salt of pravastatin in a crystalline form according to any one of claims 2 to 5

20. A pharmaceutical formulation of the sodium salt of pravastatin in a crystalline form, wherein the crystals in an X-ray diffraction measurement produce a signal sufficiently comparable to that illustrated in the diffractogram shown in Figure 2.

21. Use of a crystalline sodium salt of pravastatin for the preparation of pharmaceutical products for the treatment of hypercholesterolemia and hyperlipidemia.

Description

MICROBIAL PROCESS FOR PREPARING PRAVASTATIN

FIELD OF THE INVENTION

The present invention relates to microbial processes for the preparation of pravastatin.

BACKGROUND OF THE INVENTION

Hypercholoesterolemia has been recognized as a major risk factor for atherosclerotic disease, specifically for coronary heart disease. Biosynthesis of cholesterol is a major contributing factor to hypercholesterolemia. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate in the rate determining step in the biosynthesis of cholesterol. During the past two decades, 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMG-CoA reductase EC. 1.1.1.34) has been extensively studied.

Mevinolin and related compounds biosynthesized by different fungal species have been found to be competitive inhibitors of this enzyme [Endo, A. et al., J. Antibiotics 29,13461348 (1976): Endo. A. et al., FEBS Lett. 72,323-326 (1976): Kuo, C. H. et al., J. Org.

Chem. 48,1991-1998 (1983)].

Pravastatin is a member of this family of HMG-CoA reductase inhibitors, along with compactin, lovastatin, simvastatin, fluvastatin and atorvastatin. Pravastatin was first isolated as a minor canine metabolite of compactin (Tanaka, M. et al., unpublished) in the course of metabolic studies of compactin [Aral, M. et al., Sankyo Kenkyusho Nempo, 40, 1-38 (1988)].

Tissue selectivity is a unique characteristic of pravastatin. Pravastatin selectively inhibits cholesterol synthesis in the liver and small intestine but only weakly inhibits cholesterol synthesis in other organs, Koga, T. et al. Biochim. Biophys. Acta, 1990,1045, 115-120. Pravastatin has an advantage of lower toxicity than the other HMG-CoA reductase inhibitors.

It has been reported that compactin can be converted to pravastatin by microbial hydroxylation using various genera of fungi as well as bacteria belonging to the genera
Nocardia, of the group Actinomycetes; the genera Actinomudura, of the group
Maduromycetes and the genera Streptomyces roseochromogenes and Streptomyces carbophilus, among other species of the group Steptomyces (U. S Patent No. 5,179,013,
U. S. Patent No. 4,448,979, U. S Patent No. 4,346,227, U S Patent No. 4,537,859,
Japanese Patent No. 58-10572).

A problem is encountered with the use of fungi for the production of pravastatin.

Fungi generally do not tolerate high loads of compactin added in the culture medium, presumably due to the antifungal activity of compactin [Serizawa, N et al., J. Antibiotics 36,887 891 (1983)]

The cytochrome P450 system has been shown to be required for the hydroxylation of compactin to pravastatin by Streptomyces carbophilus bacteria [Matsuoka, T. et al

Eur. J. Biochem. 184,707-713 (1989)]. A problem with the use of the cytochrome P450 system is that recombinant DNA manipulations of it are difficult because it is a complex of proteins rather than a single protein.

There is a need for an improved microbial process for preparing pravastatin that can tolerate high concentrations of compactin and produce pravastatin in high yield and at high concentration in the fermentation broth.

SUMMARY OF THE INVENTION

The present invention provides a new microbial process for the preparation of pravastatin. More particularly, this invention provides a microbial process for the preparation of pravastatin of formula (I)

from a compound of the general formula (II)

wherein R+ stands for an alkali metal or ammonium ion, with a prokaryote from genus Micromonospora of the Actinoplaneles group able to hydroxylate a compound of the general formula (II) at the 6 (3 position.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new microbial process for the preparation of pravastatin.

The present invention is the culmination of an investigation undertaken to find a microorganism that would produce pravastatin at higher concentrations and under more advantageous conditions than has been possible with known microbial systems. Over 6,000 actinomycete strains were screened. Of these, only ten microorganisms were found to be capable of hydroxylating the sodium salt of compactin to produce pravastatin. In particular, the following species had this capacity: Streptomyces violaces No. 1/43 (Kampfer et al. 1991), Streptomyces rochei No 1/41 (Berger et al. 1989), Streptomyces resistomycificus No. 1/44 (Lindenbein 1952), Streptomyces fanatus (Frommer 1959), Streptomyces sp. No. 1/28, Micromonospora sp. No. IDR-P3, Micromonospora purpurea No. IDR-P4 (Luedemann and Brodsky 1964), Micromonospora megalomicea ssp. nigra No. IDR-P6 (Weinstein et al 1969), Micromonospora rosaria No. IDR-P7 (Horan and Brodsky 1986). Since it was not previously known that species of the Micromonospora genus were able to convert salts of the acid form of compactin into pravastatin, we undertook a detailed study the Micromonospora species that screened positive.

Micromonospora is a genus belonging to the actinomyceles taxonomic group of bacteria. Within the order Actinomycetales and the suprageneric group of Actinoplanetes, the genus Micromonospora has been shown to be more closely related to sporangiaforming actinomyceles, such as Actinoplanes and Dactylosporangium, and sharply distinct from other monosporic genera such as Thermomonospora and Thermoactinomyces, with which it was has been associated. The genera of Actinoplanetes have similar chemotaxonomic characters and nucleic acid affinities. They are Gram-positive, non-acid fast organisms growing with nonfragmenting, branched and septate hyphae of 0.2-1.6 um in diameter. Aerial mycelium is rarely developed or only sparse. Genus Micromonospora rskov, 1923.

Micromonospora chalcea (Foulerton, 1905) form well-developed, branched, septate mycelium averaging 0.5 um in diameter. Nonmotile spores are formed singly, sessile, or on short or long sporophores that often occur in branched clusters. Sporophore development is monopodial or in some cases sympodial. Aerial mycelium is absent or in some cultures appears irregularly as a restricted white or grayish bloom. Cell walls contain meso diaminopimelic acid and/or its 3 hydroxy derivative and glycine. Xylose and arabinose are present in cell hydrolysates. Characteristic phospholipids are phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides.

Micromonospora chalcea are aerobic to microaerobic and are chemoorganotrophic. They are sensitive to pH below 6.0 Growth occurs normally between 20 C and 40 C but not above about 50 C. rskov, 1923.

It has been observed that several significantly different species of the genus Micromonospora are able to hydroxylate compactin at the 6p-position and, thus, it appears that the ability to hydroxylate compactin at the 60-position is widely shared by species of genus Micromonospora. The Micromonospora of the present invention include wild type and mutant strains that are capable of converting a compactin substrate to pravastatin. Preferred Micromonospora used to further describe certain preferred embodiments of the invention and to illustrate it with specific examples were selected for their high hydroxylating capacity, which can exceed about 90% at 0.1 g/liter concentration of compactin acid sodium salt. The following strains of Micromonospora were deposited on April 13,1999 at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001271 of Micromohospora purpurea IDR-P4; NCAIM P (B) 001272, Micromonospora echinospora ssp. echinospora IDR-P6; NCAIM P (B) 001273, Micromonospora meg alomicea ssp. nigra IDR-P6; and NCAIM P (B) 001274 of Micromonospora rosaria IDRP7
An isolated Micromonospora species, numbered IDR-P3, was deposited on October 13,1998 at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM P (B) 001268. Strain No.

IDR-P3 of Micromonospora sp. was isolated from a mud sample of Lake Balaton, Hungary. In addition to producing pravastatin from compactin sodium salt in high concentration under conditions suitable to large scale fermentation, this species biosynthesizes only minor amounts of other structurally related compounds. Thus this species is very well adapted for the industrial production of pravastatin.

The taxonomic features of the cultures of Micromonospora IDR-P3 are summarized as follows.

Micromorphological properties: Substrate mycelium is composed of well developed, more curved than straight, branching filaments. In slide cultures, the monopodial system of branching hyphae (sporophores) may be observed. Spores are single, spherical, approximately 1.8 ure in diameter and are dispersed evenly on hyphal filaments. Spores are either sessile or on the end of short sporophores. In broth cultures, spores were not observed on sporulating hyphae, possibly because of the mature spores are released rapidly into the medium.

Cultural morphological properties:
Czepak-sucrose agar : Medium growth, the colonies are of reddish color covered by point-like black sporulating areas.

Glucose-asparagine agar: The growth was recorded as point-like and elevated, reddish-brown or black colonies. Reddish diffusible pigment.

Nutrient agar: Fair growth, elevated, reddish-brown or black colonies. Reddishbrown exopigment in the medium.

Yeast extract-malt extract agar (ISP Med. 2): Well developed, elevated and wrinkled, brown colonies, covered partly with black sporulating areas or with"pseudoaerial mycelium"appearing as a restricted whitish or greyish bloom. Brownish or brownish-red soluble pigment.

Inorganic salts-starch agar (ISP Med. 4): Medium growth of reddish-brown elevated and wrinkled colonies. Light reddish soluble pigment

Glycerol asparagine agar (ISP Med. 5): Growth only in traces, off-white or light orange colored, flat colonies, light rose soluble pigment

On some media observing soluble pigment has a particular indicator character being yellow in the acid pH range and in the basic pH range slightly turns

into dark shade of reddish color.

Carbon source utilization: Godd growth on and positive utilization of L-arabinose, Dcellobiose, D-fructose, D-glucose, lactose, D-maltose, D-mannitol, D-rmannose, a-methyl
D-glucbside, L-rhamnose, D-ribose, D-sucrose, D-trehalbse and D-xylose. Adonitol, dulcitol, myo-Indsitol, inulin, D-melezitose, D-raffinose are not utilized Growth with Dgalactose, glycerol, D-melibiose and D-salicin was slightly better than on the negative control medium.

Nitrogen source utilization: Good growth with yeast extract and NZ-Amine, no utilization of L-asparagine, L-glutamic acid, NH4NO3 and NaNO3.

Other physiological-biochemical properties: Cellulose and starch are hydrolyzed, milk is digested strongly. Nitrate reduction test is negative. No growth on potato slices without calcium carbonate (pH 5.8-6.0).

A preferred form of the invention, base upon our studies of the Micromonospora strains deposited with the National Collection of Agricultrual and Industrial
Microorganisms, Budapest, Hungary, relates to a new microbial process for the preparation of pravastatin of formula (I)

from a compound of general formula (II),

wherein R+ stands for an alkali metal or ammonium ion, by the submerged cultivation of a strain able to 6p-hydroxylate a compound of formula (II) by aerobic fermentation and by the separation and purification of the compound of formula (I) formed in the course of the bioconversion wherein the process comprises the steps of : a) cultivating a microorganism of the genus Micromonospora able to 6p-hydroxylate a compound of formula (II)- wherein R+ is defined above-in a nutrient medium containing assimilable carbon and nitrogen sources and mineral salts at 25-32 C, thereafter b) feeding the substrate until the end of bioconversion, c) fermenting the substrate until the end of bioconversion, then d) separating the compound of formula (I) from the culture broth and, if desired, purifying the same.

According to a yet more preferred embodiment, pravastatin is produced from either a wild strain or mutant strain of Micromonospora selected from the group consisting of Micromonospora purpurea IDR-P4 [NCAIM P (B) 001271],
Micromonospora actinospora ssp. echinospora IDR-P6 [NCAIM P (B) 001272],
Micromonospora megalomicea ssp. nigra IDR-P8 [NCAIM P (B) 001273] and
Micromonospora rosaria IDR-P7 [NCAIM P (B) 001274]. According to the most preferred embodiment of the invention, pravastatin is produced with Micromonospora sp.

IDR P3 [NCAIM P (B) 001268].

The present invention can be carried out by in situ fermentation, that is, by hydroxylation conducted in the presence of actively growing microorganisms using batch culture or fed-batch culture techniques

The hydroxylation may be conducted by employing agitation, such as in shakeflask culture, or aeration and agitation in fermentors, when the compound of the formula (II) is added to the growing cultures. In such cases an anti foaming agent may be employed.

The microorganisms may be cultivated and maintained using an appropriate nutrient medium containing carbon and nitrogen sources and inorganic salts and trace elements. Exemplary assimilable carbon sources include glucose, glycerol, dextrin, starch, rammose, xylose, sucrose, soluble starch, etc. Exemplary assimilable nitrogen sources include soybean meal, corn steep liquor, peptop, yeast extract, meat extract, ammonium citrate, ammonium

sulfate, etc. Inorganic salts such as calcium carbonate, sodium phosphates, potassium phosphates etc., may also be added to the culture medium.

Preferred media for the growth of microorganisms are described in the examples.

Preferably the culture is an agitated liquid medium. The preferred temperature range for conducting the hydroxylation is from about 25 C to 37 C, most preferably about 25 C to 32 C. The preferred pH is from about 6.0 to 9.0, most preferably between about 7.0 to 8.5. The preferred shaking condition is about 200 rpm to 400 rpm, most preferably about 250 rpm.

Any compactin concentration can be used that will result in production of pravastatin. A compactin concentration of between about 0.1 and 10 g/liter, more preferably between about 0.3 and 3.0 g/liter, is well suited for in situ hydroxylation. The percentage of conversion of compactin to pravastatin is not a critical feature of the inventive process. However, conversion preferably occurs to the extent of about 30% or more, preferably about 60% or more and yet more preferably about 90% or more.

The composition of the fermentation broth may be monitored by high performance liquid chromatographic method (HPLC) using conditions described in the Examples.

Pravastatin can be isolated from the fermentation broth by any method, e. g., extraction-reextraction, anion exchange chromatography or precipitation. The following isolation processes are well suited to isolating pravastin as a biosynthetic product of Micromonospora. However, these processes are provided for the sole purpose of completely disclosing the favored modes of obtaining pravastatin starting from compactin and a strain of the genus Micromonospora and are not intended to limit the invention in any way.

After finishing the bioconversion, pravastatin can be extracted either from the fermentation broth or from the filtrate obtained after the separation of the bacterium cells.

Bacterium cells can be removed either by filtration or centrifugation. However, it is advantageous, especially in an industrial scale, to perform a whole broth extraction.

Extraction solvents are any solvent that is not wholly miscible with water. Preferred extraction solvents have low solubility in water. Especially preferred solvents include acetic acid esters having a 2-4 carbon atom containing aliphatic alkoxyl moiety, such as ethyl acetate and isobutyl acetate.

In the course of our experiments it was recognized that pravastatin can be precipitated from an organic extract of the broth as a crystalline salt with secondary amines. Further, it was found that several secondary amines containing alkyl-, cycloalkyl , aralkyl-or aryl-substituents are especially well suited for the salt formation. Among these, the following secondary amines are the most preferred, in part because of their low toxicity: dioctylamine, dicyclohexylamine and dibenzylamine.

The method of isolating the organic secondary amine salt of pravastin is illustrated with dibenzyl amine. Isolation of the dibenzylamine salt is carried out by adding dibenzylamine in 1.5 equivalent quantity related to the pravastatin content of the extract, then the extract is concentrated by vacuum distillation to 5% of its original volume, then another quantity of dibenzylamine is added into the concentrate in 0.2 equivalent ratio. The crystalline dibenzylamine salt is precipitated from the concentrate.

The crystalline crude product is filtered and dried under vacuum, and is clarified with charcoal in methanol or acetone solution. Pravastatin dibenzylamine salt can be further purified by recrystallization from acetone.

Pravastatin organic secondary amine salts can be transformed to pravastatin with sodium hydroxide or sodium alkoxide. A preferred sodium alkoxide is sodium ethoxide.

The isolation of pravastatin via a secondary amine salt intermediate is a simpler procedure than any of the previously known isolation procedures. During the procedure, artifacts are not formed. Separation of pravastatin from by-products of the bioconversion and from the various metabolic products biosynthesized by the hydroxylating microorganism can be advantageously solved.

Another process for isolating pravastatin from the fermentation broth takes advantage of the fact that the bioconversion produces pravastatin in its acidic form.

Thus, pravastatin can be isolated from the broth by adsorption on an anion exchange resin column, preferably from a filtrate of the broth. Strongly basic anion exchange resins like a polystyrene-divinylbenzene polymer carrying quaternary ammonium active groups such as Dowex'Al 400 (OH-form), Dowexh 1x2 (OH-form), DowexZ 2x4 (OH-form), Amberlite IRA 900 (OH-form) resins are well suited for absorbing pravastatin free acid from the broth. The material that absorbs on the ion exchange resin can be eluted from the column by aqueous acetic acid or a mixture of acetone and water containing sodium chloride. A 1 % solution of sodium chloride in a (1: 1) acetone: water mixture is a particularly preferred eluent. Pravastatin-containing fractions are combined and the acetone is distilled off under vacuum. The pH of the concentrate is adjusted with 15% sulphuric acid to a range of 3.5-4.0 and the acidified aqueous solution is extracted with ethyl acetate. Pravastatin can be re-extracted from the ethyl acetate extract using a 1/10 to 1/20 volume ratio of 5% sodium hydrogen carbonate or other mildly alkaline basic solution (pH 7.5-8.0).

Pravastatin can be recovered from the alkaline aqueous extract in a pure form by column chromatography on a non-ionic adsorption resin. In one method, any residual ethyl acetate that dissolved in the alkaline aqueous phase during extraction should be removed by vacuum distillation and then the aqueous extract is loaded on a Diaion HP-20 column. Pravastatin adsorbed on the column is purified by elution with aqueous acetone in which the acetone content is gradually increased, then the chromatographic fractions containing pravastatin as a single component are combined and concentrated under vacuum. The concentrate is clarified with charcoal and lyophilized. The pravastatin is then crystallized from an ethanol-ethyl acetate mixture, affording pravastatin in a quality acceptable for pharmaceutical application.

Another method for isolating pravastatin lactonizes pravastatin to improve separation from other acidic organic substances in the broth. Before extraction, the pH of either the fermentation broth or the filtrate of the broth is adjusted to 3.5-3.7 with a mineral acid, preferably with dilute sulphuric acid. The broth is then extracted with a water-immiscible organic solvent, preferably an acetic acid ester with a 2-4 carbon atom containing aliphatic alkoxyl moiety, such as ethyl acetate or isobutyl acetate. The ethyl acetate extract is washed with water and dried with anhydrous sodium sulphate. Then, pravastatin is converted to its lactone. The lactone ring closure may be carried out in dried ethyl acetate solution at room temperature under continuous stirring and using a catalytic amount of trifluoroacetic acid. Lactone ring closure can be monitored by thin layer chromatography ("TLC"). After the lactone has formed, the ethyl acetate solution is washed with 5% aqueous sodium hydrogen carbonate solution and then with water. The ethyl acetate solution is dried with anhydrous sodium sulphate and ethyl acetate is evaporated under vacuum. The residue is purified with silica gel column chromatography eluting with mixtures of ethyl acetate and hexane and gradually increasing the ethyl acetate content.

The purified pravastatin lactone is converted to pravastatin sodium by hydrolysis at room temperature in ethanol with an equivalent or more of sodium hydroxide. After the pravastatin sodium salt has formed, the pravastatin sodium can be precipitated with acetone. The precipitate is filtered and washed with acetone and n hexane and dried under vacuum. The pravastatin sodium can be crystallized from an ethanol-ethyl acetate mixture to yield pravastatin sodium in a quality acceptable for pharmaceutical application

Another method of isolating pravastatin uses chromatography on Sephadex LH 20 gel. Pravastatin exceeding the purity of 99.5% (measured by HPLC) can be produced by chromatography on Sephadex LH 20 gel

Having thus described the invention with respect to certain preferred embodiments, the inventive processes for biosynthesis of pravastatin using Micromonospora and isolating pravastatin will further be illustrated with the following examples.

EXAMPLES

High performance liquid chromatography ("HPLC") was performed using equipment manufactured by Waters@. HPLC conditions: column packing Waters
Novapack C18 5pm reverse phase packing; UV detection: k = 237 nm; injection volume: 10 pl; flow rate: 0.6-0.9 ml/min linear gradient; gradient elution: solvent A = acetonitrile0.1M NaH2PO4 in water (25: 75), solvent B = acetonitrile-water (pH 2 with H3PO4) (70: 30). The gradient program is shown in Table 1.

Table 1
Time (min) Flow rate (ml/min.) Eluent A (%) Eluent B (%)
0 0.6 100 0
2 0.7 100 0
20 0.9 0 100
21 0.9 0 100
22 0.9 100 0 27 0. 7 100 0
Retention times: pravastatin (Na salt) 10.6 min; compactin (acid form) 19.5 min; pravastatin (lactone form) 17.3 min, compactin (lactone form) 23 5 min.

Example 1
A soluble starch agar medium ("SM", Table 2) was adjusted to a pH of 7.0 and then sterilized at 121 C for 25 minutes.

Table 2
Composition of SM medium
Soluble starch 10.0 g
Yeast extract 5.0 g
Na2HPo4 1.15 g
KH2PO4 0.25 g
KCl 0.2 g
MgSO4-7H2O 0 2 g
Agar 15.0 g
Water 1000 ml
The SM medium was then innoculated with Micromonospora sp. IDR-P3 [NCAIM P (B) 001268]. A spore suspension in distilled water (5 ml) was prepared from spores obtained from the 7-10 day old, soluble starch agar (SM) slant culture of Micromonospora sp. IDR-P3 [NCAIM P (B) 001268]

The suspension was used to inoculate T1 inoculum medium (100 ml, Table 3) in a 500 ml Erlenmeyer flask after adjusting the pH of the T1 medium to 7 0 and sterilization at 121 C for 25 minutes

Table 3
Composition of T1 medium
Soluble starch 20 0 g
Yeast extract 10 0 g

CaCO3 5.0 g
COC'2-6H20 2.0 mg
Water 1000 ml
The culture was shaken on a rotary shaker (250 r. p. m.; amplitude: 2.5 cm) for 3 days, at 32 C. Then, 5 ml portions of this inoculum culture were used to inoculate ten 500 ml Erlenmeyer flasks each containing TT medium (100 ml, Table 4) that had been adjusted to pH 7.0 and sterilized at 121 C for 25 minutes.

Table 4
Composition of TT medium
Potato starch 30.0 g
Soybean meal 30.0 g
CaCO3 5.0 g
Cocl2*6H20 2.0 mg
Palm oil 2.0 g
Water 1000 ml
The bacteria were incubated at 32 C for 72 hours. The sodium salt of compactin (50 mg) was then added to each flask in distilled water, the bioconversion was continued at 32 C for a further 96 hours. The conversion of compactin sodium salt to pravastatin measured 82% by HPLC.

Key point

After finishing the fermentation, the cultures were combined. Pravastatin formed in an average concentration of 410 pg/ml. Pravastatin was isolated as follows. The fermentation broth was centrifuged at 2500 r. p. m. for 20 min. The supernatant of the broth and the cells of bacterium were separated. Water (250 ml) was added to the cells of bacterium and the suspension was stirred for one hour and filtered. The supernatant and filtrate were combined. The pH was adjusted to 4.0 with 15% sulphuric acid. The acidic filtrate/supernatant mixture was extracted with ethyl acetate (3x300 ml). The combined ethyl acetate extracts were washed with water (300 ml), dried with anhydrous sodium sulphate and concentrated under vacuum to 100 ml volume.

Pravastatin lactone was prepared from pravastatin by adding trifluoro acetic acid in catalytical amount at room temperature with continuous stirring. Formation of pravastatin lactone was monitored by TLC: adsorbent: Kieselgel (silica gel) 60 F254 DC (Merck) on aluminium foil backing; developing solvent: acetone: benzene: acetic acid (50: 50: 1.5) mixture; detection: phosphomolybdic acid reagent; Rf (pravastatin lactone) = 0.7. After lactonization was complete, the ethyl acetate was washed with 5% aqueous sodium hydrogen carbonate (2x20 ml), then water (20 ml), and dried with anhydrous sodium sulphate. Ethyl acetate was evaporated under vacuum. The residue (0.5 g) was separated by gradient column chromatography on 10 g of Kieselgel 60 adsorbent (column diameter: 1.2 cm) eluting with ethyl acetate-n-hexane mixtures of increasing polarity.

Pravastatin lactone was eluted from the column with a mixture of 60% ethyl acetate/nhexane. The fractions containing pravastatin lactone were combined and evaporated under vacuum. The residue (230 mg) was dissolved in ethanol (5 ml) and then 110 mole % of sodium hydroxide was added as a 1M ethanolic solution with stirring. Stirring was continued for half an hour at room temperature. The solution was then concentrated to 2 ml volume. Acetone (4 ml) was added to the concentrate. The mixture was kept at +5 C overnight. The precipitate was filtered, washed with acetone (2 ml) and then n-hexane (2 ml) and dried under vacuum at room temperature. The resulting crude pravastatin was dissolved in ethanol. The solution was clarified with charcoal and then pravastatin (170 mg) was crystallized from ethanol-ethyl acetate mixture.

Characterization
Melting point: 170-173 C (decomp.)
[a] D20 +156 (c: 0,5, in water)

Ultraviolet absorption spectrum (20, ug/ml, in methanol): SmaX = 231,237,245 nm (logs =4.203, 4.136)

Infrared absorption spectrum (KBr): v OH 3415, v CH 2965, v C-0 1730, v COO- 1575 cm-1.

1H-NMR spectrum (D2O, δ, ppm): 0.86, d, 3H (2'-CH3); 5.92, dd, J = 10.0 and 5.4 Hz, 1H (3-H); 5.99, d, J = 10.0 Hz, 1H (4-H); 5.52, br, 1H (5-H); 4.24, m, 1H (6-H); 5.34, br, 1H (8-H); 4.06, m, 1H (ss-H), 3.65, m, 1H (6-H); 1.05, d, 3H (2'-CH3); 0.82, t, 3H (4'H3).

13C-NMR spectrum (D2O, δ, ppm): 15.3, q (2-CH3); 139.5, d (C-3); 129.5, d (C4); 138.1, s (C-4a); 127.7, d (C-5); 66.6, d (C-6), 70.1, d (C-8); 182.6, s (COO-); 72.6, d (C-ss); 73.0, d (C-6); 182.0, s (C-1'); 18.8, q (2'-CH3); 13.7, q (C-4').

Positive FAB mass spectrum (characteristic ions): 469 [M+Na] +; 447 [M+H] +.

Negative FAB mass spectrum (characteristic ions): 445 [M-H]-; 423 [M-Na]-, m/z 101 [2-methyl-bulyric acid-].

Example 2
Bioconversion medium MT (Table 5) was adjusted to pH 7.0 and sterilized at 121 C for 25 minutes.

Table 5
Composition of MT Bioconversion Medium
Potato starch 10.0 g
Dextrose 20.0 g
Soybean meal 10.0 g
Yeast extract 10.0 g
CaCO3 5.0 g
COC'2-6H20 2.0 mg
Sunflower oil 2.0 g
Water 1000 ml
Ten 500 ml Erlenmeyer flasks each containing MT bioconversion medium (100 ml) were inoculated with the inoculum culture prepared in Example 1 and incubated at 28 C for 96 hours. The sodium salt of compactin (50 mg) was dissolved in a minimum of distilled water and added to each flask. Fermentation was continued for 72 hours. Then another 50 mg of compactin sodium salt in distilled water was added to each of the cultures and the fermentation was continued for another 72 hours.

The cultures were combined and pravastatin was isolated from the broth by the following procedure. The combined cultures, containing 750 mg of pravastatin according to the HPLC assay, were centrifuged at 2500 r. p. m. for 20 min. The separated cells of bacterium were stirred with water (250 ml) for an hour, then filtered. The supernatant and filtrate were combined and the pH of the resulting solution was adjusted to 3.5-4.0 with 15% sulphuric acid. The solution was extracted with ethyl acetate (3x300 ml). Then 150 mole% of dibenzylamine--calculated for the pravastatin content--was added to the ethyl acetate extract. The ethyl acetate extract was evaporated to about 30 ml volume and the suspension was kept overnight at 0-5 C. Precipitated pravastatin dibenzylammonium salt was filtered and washed on the filter with cold ethyl acetate and n-hexane and dried under vacuum. The crude pravastatin dibenzylammonium salt (1.1 g) was dissolved in acetone (33 ml) at 62-66 C. The solution was clarified with charcoal (0.1 g) for half an hour. The charcoal was removed by filtration from the solution and washed with warmed acetone (10 ml). Crystals precipitated from the concentrate and were dissolved again at 62-66 C. The solution was kept at +5 C overnight. The precipitate was filtered, washed with cold acetone and n-hexane and dried under vacuum. The pravastatin dibenzylammonium salt so obtained (0.7 g) was suspended in ethanol (10 ml), then 110 mole% of sodium hydroxide was added to the solution as a 1M aqueous solution. Stirring of the alkaline solution was continued for half an hour at room temperature. Water (10 ml) was added

and the pH of the solution was neutralized. The ethanol was distilled off under vacuum. The resulting aqueous concentrate was separated by gradient column chromatography on a column filled with 50 ml of Diaion HP 20 resin (column diameter: 1.5 cm). The column was eluted with acetone-deionized water mixtures, increasing the concentration of the acetone in 5% increments. Pravastatin could be eluted from the column with a 15% acetone-deionized water mixture. Fractions were analysed by the
TLC method given in the Example 1: Rf (pravastatin) = 0.5. Fractions containing pravastatin were combined and the acetone was evaporated under vacuum.

Lyophilization of the aqueous residue gave chromatographically pure pravastatin (390 mg).

Example 3
TT/2 medium (4.5 L, Table 6) was sterilized at 121 C for 45 minutes in a laboratory fermentor and inoculated with the Micromonospora sp. IDR-P3 inoculum shake culture in T1 medium (500 ml) prepared as described in Example 1.

Table 6
Composition of TT/2 Bioconversion Medium
Glucose 75.0 g
Soluble starch 50.0 g
Soybean meal 50.0 g
Yeast extract 50.0 g
soya peptone 5.0 g
CoCl2H20 2.0 mg
CaCO3 5.0 g
Water 1000 ml
The medium was then incubated at 28 C, aerated with 150 L/h of sterile air and stirred with a flat blade stirrer at 300 r. p. m. The fermentation was continued for 72 hours and the sodium salt of compactin (2.5 g) was added to the culture. By the 48""hour of the bioconversion the compactin substrate was consumed from the fermentation broth.

Additional compactin sodium salt (2.5 g) was added to the culture. The second dose of compactin substrate was consumed in 24 hours. The conversion rate of compactin sodium salt into pravastatin was 90%.

Example 4
TT/1 fermentation medium (4.5 L, Table 7) was adjusted to pH 7.0 and sterilized at 121°C for 45 minutes in a laboratory fermentor

Table 7
Composition of TT/1 Bioconversion Medium
Glucose 125.0 g
Potato starch 25.0 g
Soybean meal 50.0 g
Yeast extract (Gistex) 50.0 g
soya peptone 50.0 g
COC'2 6H20 2.0 mg
CaCO3 5.0 g
Sunflower oil 2.0 g

Water 1000 ml
The TT/1 medium was inoculated with the Micromonospora sp. IDR-P3 inoculum shake culture (500 ml) prepared as described in Example 1. The culture was then incubated at 28 C, aerated with 200 L/h of sterile air and stirred with a flat blade stirrer at 400 r. p. m. for 96 hours. The sodium salt of compactin (2.5 g) was added to the culture as a sterile filtered aqueous solution. The fermentation was conducted at 28 C. By the fifth day of fermentation the compactin was consumed from the fermentation broth.

Additional compactin sodium (7.5 g) was added in 2.5 g portions intermittently over two days. The additional compactin sodium salt was completely converted to pravastatin within four days of the first addition. At the end of the fermentation, compactin sodium salt (10 g) was converted to pravastatin (9 g, 90%).

Pravastatin at a concentration of 1800 ug/ml was isolated from the broth as follows. The culture broth (5 L) was centrifuged at 2500 r. p. m. for 20 min and the supernatant was separated from the cells of the bacterium. Water (2 L) was added to the separated cells and the resulting suspension was stirred for one hour and filtered. The supernatant and filtrate were combined and passed through a column containing Dowex
AI 400 (OH-) resin (300 g, column diameter: 4 cm) at a flow rate of 500 ml/hour. The resin bed was washed with deionized water (1 L). The column was then eluted with a 1: 1 acetone-water mixture (1 L) containing 10 g of sodium chloride, collecting in 50 ml fractions. The fractions were analyzed by the TLC method given in the Example 1.

Fractions containing the product were combined and the acetone was distilled off under vacuum. The pH of the concentrate was adjusted to 3.5-4.0 value with 15% sulphuric acid. The concentrate was extracted with ethyl acetate (2x250 ml). Deionized water (40 ml) was added to the combined ethyl acetate extracts. The pH of the aqueous phase was adjusted to 7.5-8.0 with 1M sodium hydroxide. After 15 min stirring, the aqueous and ethyl acetate phases were separated. The aqueous alkaline extraction was twice repeated.

The combined alkaline aqueous solutions were concentrated to 50 ml volume and the residue was separated by chromatography over Diaion HP20 (Mitsubishi Co. Japan, 600 ml, column diameter 3.8 cm). The column was washed with deionized water (600 ml), then eluted with acetone-deionized water mixtures, increasing the concentration of acetone in the eluent in 5% increments. The eluent was collected in 50 ml fractions. The eluent was analysed by the TLC method given in the Example 1. Pravastatin was eluted from the column in the 15% acetone-deionized water mixture. Fractions containing pure pravastatin as determined by TLC were combined and the solution was concentrated under vacuum to a volume of 150 ml. The concentrated eluent was clarified by stirring over charcoal (0.6 g) at room temperature for 1 hour. The charcoal was filtered off and the filtrate was lyophilized. The resulting lyophilised pravastatin (6.5 g) was crystallized twice from a mixture of ethanol and ethyl acetate. The precipitate was filtered and washed with ethyl acetate (20 ml) and n-hexane (20 ml), and dried under vacuum at room temperature to obtain chromatographically pure pravastatin (4.6 g).

Example 5
The sterile soluble starch medium SM of Example 1 was innoculated with Micromonospora echinospora ssp. echinospora IDR-P5 [NCAIM P (B) 001272] bacterium strain and incubated for ten days. A spore suspension in distilled water (5 ml) was prepared from spores obtained from the ten day old soluble starch medium and the suspension was used to inoculate 100 ml of the sterile T1 inoculum medium described in
Example 1 in a 500 ml Erlenmeyer flask. The culture was shaken on a rotary shaker (250 r. p. m., 2.5 cm amplitude) for 3 days at 28 C. Then, 5 ml portions of the obtained culture were transferred to ten 500 ml Erlenmeyer flasks, each containing 100 ml of bioconversion media TT/1 that had been sterilized by heating to 121 C for 25 min. The composition of the TT/1 medium is described in Example 3. Flasks were incubated with shaking on a rotary shaker (250 r. p. m., 2.5 cm amplitude) for 3 days at 25 C. Compactin sodium salt (10 mg) was added as a sterile filtered aqueous solution to each of the flasks.

Fermentation was continued for 168 hours at 25 C. At the end of the bioconversion, the pravastatin content of the fermentation broth was 10 mg/ml as

determined by HPLC.

Example 6
Inoculation, incubation, fermentation and substrate feeding were carried out with the Micromonospore megalomicea ssp. nigra IDR-P6 [NCAIM P (B) 001273] bacterium strain as described in Example 5. The pravastatin content of the fermentation broth after 168 h was determined to be 50 pg/ml by HPLC.

Example 7
An inoculum culture of the Micromonospora purpurea IDR-P4 [NCAIM P (B) 001271] bacteria strain (5 ml) was prepared according to the method described in
Example 1. The inoculum culture was used to seed TT/14 medium (100 ml, Table 8) in 500 ml Erlenmeyer flasks after adjustment of the pH of the TT/14 medium to 7.0 and sterilization at 121°C for 25 min.

Table 8
Composition of TT/14 Bioconversion Medium
Potato starch 5.0 g
Glucose 25.0 g
Yeast extract (Gistex) 15.0 g
soya peptone 15.0 g
CaCO3 5.0 g
CoCl2&commat,6H20 2.0 mg
Tap water 1000 ml
The flasks were shaken on a rotary shaker (250 r. p. m., 2.5 cm amplitude) for 3 days. Compactin sodium salt feeding, bioconversion and determination of the pravastatin content were carried out as described in Example 5. At the end of the bioconversion the pravastatin content of the fermentation broth was 4011g/ml, as measured by HPLC.

Example 8
Inoculation, incubation, fermentation and compactin sodium salt feeding were carried out with the Micromonospora rosaria IDR-P7 [NCAIM P (B) 001274] bacterium strain following the method described in Example 1. At the end of the bioconversion, 350 pg/ml pravastatin was in the fermentation broth, as measured by HPLC.

Having thus described the invention with reference to certain preferred embodiments and with examples, those skilled in the art will appreciate variations that do not depart from the spirit and scope of the invention as described above and claimed hereafter.